Regulation of cellular differentiation by tissue specific transcription factor

T. Endoh, M. Mie, Y. Yanagida, M. Aizawa, E. Kobatake
Department of Biological Information, Graduate School of Bioscience and Biotechnology,
Tokyo Institute of Technology

Abstract

Tissue specific transcription factors play important roles during cellular differentiation. To regulate cellular differentiation, we paid attention to tissue specific basic helix-loop-helix (bHLH) transcription factors. In this study, we tried to regulate expression of tissue specific transcription factor for induction of cellular differentiation.

To regulate expression of transcription factor, we constructed electrical stimulation induced artificial gene expression system. Using this system, neural differentiation of neuroblast could be induced by conditional expression of neural bHLH transcription factor NeuroD2 under the control of electrical stimulation.

1. Introduction

Among the development of individuals, cells are transformed to many type of mature differentiated cells correctly. Though, the regulation mechanism of cellular differentiation is still unknown, it is sure that intracellular transcription factor plays an important role among the differentiation, and the expression of tissue specific transcription factor is critical for determine of the cell fate. For example, there are some neural basic helix-loop-helix (bHLH) transcription factors, such as MASH1, MATH1 and NeuroD, which are expressed during neural development. The disruptions of these genes in mice did not lead to normal formation of brain. On the other hand, forced expression leads to formation of neurons [1]. Especially, Farah et al. reported that transiently transfection of NeuroD2 into the pluripotent mouse P19 embryonal carcinoma cells have ability to change the cells to neural morphology [2]. Moreover the transfected cells express numerous neuron specific proteins. These results suggest that tissue specific transcription factors achieve the cell differentiation. Therefore to regulate the expression of transcription factor directly enable us the regulation of cell differentiation.

In cells, many factors modulate expression of genes at multistage, including DNA methylation, promoter activation, mRNA splicing and protein modification at post translation. Almost these modulate mechanisms are triggered by chemical stimulations, such as cytokines and hormones. However, it is shown that much other extracellular stimulations also modulate genes expressions such as osmotical stress, shearing stress, heat stress and so on. The pattern of gene expression could be changed considerably in response to these stimulations. Thus to utilize these cellular gene expression modulate systems and artificial external stimulations, we would be able to regulate the expression of genes, which attracted in. So far some gene expression systems regulated by artificial stresses have been attended to. Especially, because of heat shock protein (HSP) promoter is activated by not only heat but also various physical stresses [3,4], it have been used as mediator to connect stimulations and gene expressions. However it is difficult to regulate gene expression in specific place by usual stress methods.

Electrical stimulation is also one of the physical extracellular stimulations and it has reported that increase of intracellular Ca2+, degradation of actin has appeared in response to. And in previous study, we found that electrical stimulation also could activate the HSP promoter [5]. In this electrical stimulation system, cells were cultured on the electrode and applied potential directly. Moreover, it is easy to regulate and has a possibility to stimulate in the specific place.

In this study, we tried to regulate neural differentiation by electrically stimulated gene expression of neuron specific transcription factor, NeuroD2, with the final aim to induce neural differentiation in the required place. In order to control the expression of NeuroD2 by electrical stimulation, we constructed the plasmid containing a mouse NeuroD2 gene under the HSP promoter. The plasmid was transfected into the mouse neuroblastoma cell line N1E-115. The stably transfected cells were cultured on the electrode and electrical stimulation was applied. After applying electrical stimulation, neural differentiation was evaluated by cell morphology.

2. Materials and methods

Plasmid construction

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
00 JUN 2003	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Regulation of cellular differentiation by tissue specific transcription factor	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Department of Biological Information, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
See also ADM001697, ARO-44924.1-EG-CF, International Conference on Intelligent Materials (5th) (Smart Systems & Nanotechnology)., The original document contains color images.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**6**	

To obtain the NeuroD2 gene, brain cells were acquired from naonatal mouse and cellular RNA was extracted. Total cDNA was synthesized from mouse brain mRNA using Superscript II reverse transcriptase (Invitrogene). NeuroD2 fragment was amplifide by PCR with both end of synthetic restriction site. Then the fragment was digested and inserted to pBluescript II cloning vector (Stratagene) and sequence was performed using the published sequence [1]. The fragment was picked out again and clond into pIRES2- EGFP (Clontech), resulting an expression vector pND2- IRES2-EGFP. The CMV promoter of pND2-IRES2- EGFP was replaced with HSP promoter to constract a objective expression vedtor pHSP-ND2.

Cell culture and transfection

The mouse neuroblastoma cell line, N1E-115, was obtained from the American type culture collection and maintained in Dulbeco's eagle medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), and antibiotics at 37 ^{O}C in 5 % CO2. After maintenance culture, N1E-115 cells were transfected with pHSP-ND2 by using FuGENE6 (Roche) to establish the subclonal cell line named HSP-ND2. After the transfection, cells that containing the plasmid were selected in DMEM within 500 μg/ml G418 continuously.

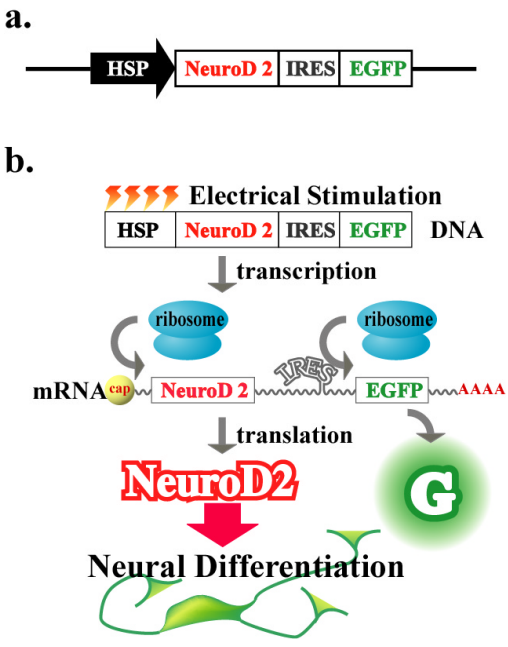

Fig. 1. Constructed plasmid sequence (a) and scheme of neural differentiation in response to electrical stimulation (b). HSP promoter was activated by electrical stimulation. Ribosome can start translate the mRNA from both cap and IRES dependent translation. EGFP fluorescent cells differentiate into neurons by the function of NeuroD2 transcription factor.

mRNA isolation and analysis

Total cellular RNA was extracted at 3 h after termination of heat shock, at 42 ^{O}C for 90 min length, using TRIzol-reagent (Gibco BRL). Total cDNA was synthesized from mRNA with oligo-dT primer, using Superscript II reverse transcriptase. Then cDNAs which coding NeuroD2 and GAPDH were amplified by PCR. The PCR products were separated by electrophoresis and the amounts were compared staining with ethidium bromide.

Applying electrical stimulation

Cells were electrically stimulated by three-electrode system, which is schematically drawn in Fig. 2. The working electrode was made of a glass plate sputtered with indium tin oxide (ITO). Counter and reference electrode were made of a platinum wire and Ag/AgCl respectively. The cells were precultured on a working electrode on which a Flexiperm-disk (Heraeus GmbH) was placed and secured tightly to the surface to form a water-tight seal. After a day culture, the cells were stimulated with applying potential to the working electrode with a potentiostat (Technical Research) through a function generator (Kenwood).

Observation of green fluorescent protein expression

The images of enhanced green fluorescent protein (EGFP) expressed in cells were obtained with an Olympus IX70 inverted fluorescent microscope. The neural differentiation was evaluated by the neurite length.

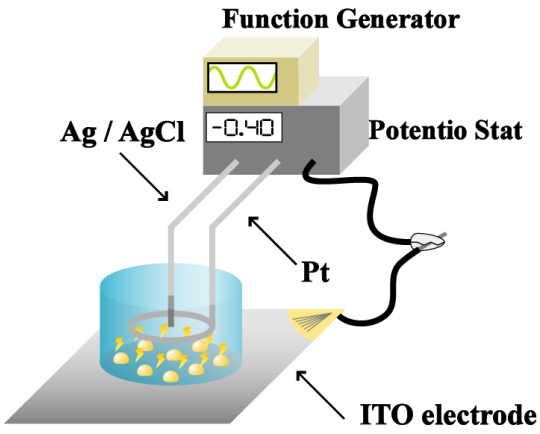

Fig. 2. Schematic drawing of electrical stimulation system. Cells are cultured on the ITO electrode and potential is applied. The potential of the ITO electrode potential is controlled with a potentiostat through a function generator.

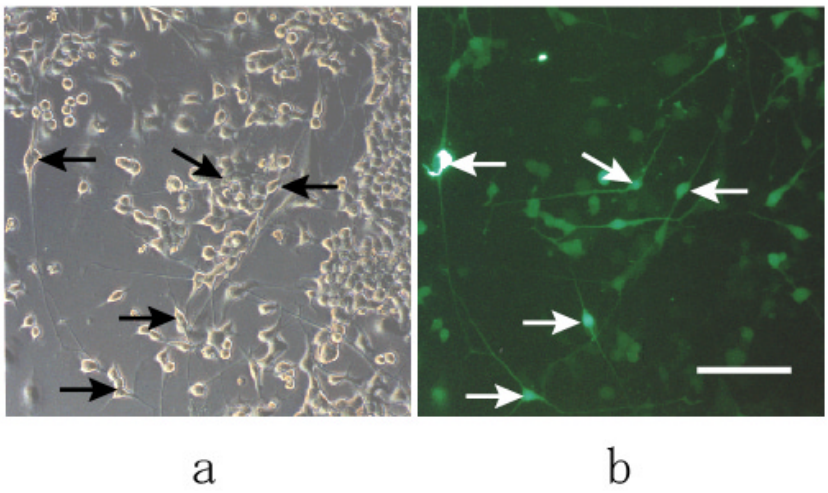

Fig. 3. NeuroD2 expression induces neural cell differentiation. N1E-115 cells were transiently transfected with pND2-IRES2-EGFP. At 5 days culture period after transfection, transfected cells expressed EGFP fluorescence and adopted neural cell morphology. The cells forming long neurites greater than 50 μm long were regarded as differentiated cells. Arrows represent differentiated cells. (a) phase contrast micro-scopy (b) fluorescence microscopy. Scale bar 200 μm.

3. Results

Induction of neural differentiation by transiently expression of NeuroD2

To investigate whether the neuron specific transcription factor NeuroD2 has ability to determine the neural differentiation in mouse neuroblastoma cell, we transiently transfected the pND2-IRES2-EGFP to N1E-115. In this plasmid, there is IRES sequence and EGFP gene followed by the NeuroD2. The mRNA, which was transcripted from CMV promoter, could be translated from both cap and IRES dependent translation. Therefore, transfected cells express two recombinant proteins and could be identified by EGFP fluorescence (Fig.1 b). After the transfection, cells start to exhibit EGFP fluorescence until 24 hours. But in this time, there was no neural morphology. However, until after 3 days some of them exhibit neurite elongation, and 5 days, almost cells that have EGFP fluorescence take neural morphology (Fig.3). In contrast, cells which were transiently transfected with pCMV-EGFP that express only EGFP were not take neural morphology and just have EGFP fluorescence. These results show that NeuroD2 gene has an ability to determine neuroblastoma cells to neural differentiation even if it were transiently expressed.

Activation of transcription in response to heat shock

HSP-ND2, which were stably transfected with pHSP-ND2, were precultured in 6 well plates. After a day, heat shock at 42 ^{O}C for 90 min were applied to cells in incubator. Total RNA was extracted from wild type N1E-115 and HSP-ND2 cells before and 3 h after heat shock, and then existence of recombinant NeuroD2 mRNA was analyzed by RT-PCR. Before the heat shock, it was not detectable both wild type and HSP-ND2 cells. Three hours later without heat shock, it was not in neither of them. However, three hours later with heat shock, it was considered in HSP-ND2 cells but not in wild type cells (Fig.4). These results indicate that transfected HSP promoter was inactive in normal cell culture, and when the external simulation, in this case the heat shock, was applied it was activated specifically.

Induction of neural differentiation in response to heat shock

Since the HSP promoter was activated in response to heat shock, HSP-ND2 cells exhibited EGFP fluorescence until 24 h after treatment of heat shock. At the time, wild type cells and HSP-ND2 without heat shock didn't exhibit EGFP fluorescence (Fig.5). HSP-ND2 cells did not take a neural morphology at 24 h later though, after 50 h neurite elongation was observed on the fluorescent cells. On the other hand, there are still neither EGFP fluorescence nor neurite morphology on wild type cells and HSP-ND2 without heat shock. These results indicate that it is possible to regulate neural differentiation mediated by recombinant NeuroD2 expression through the extracellular stimulation.

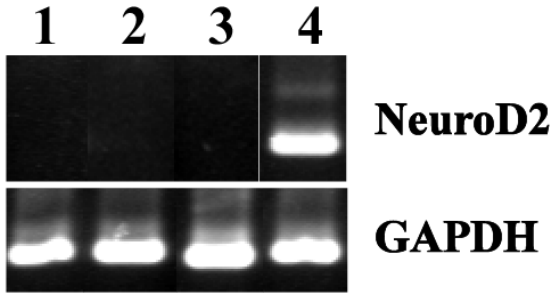

Fig. 4. Expression of NeuroD2 mRNA (top panel) and GAPDH mRNA (bottom panel) as a control in HSP-ND2 cells at 3 h after applying heat shock. Non-transfected N1e-115 cells are lanes 1 and 3. HSP-ND2 cells are lanes 2 and 4. Lanes 3 and 4 were applied heat shock at 42 ^{O}C for 90 min. Only HSP-ND2 cells with heat shock expressed NeuroD2 mRNA (lane 4)

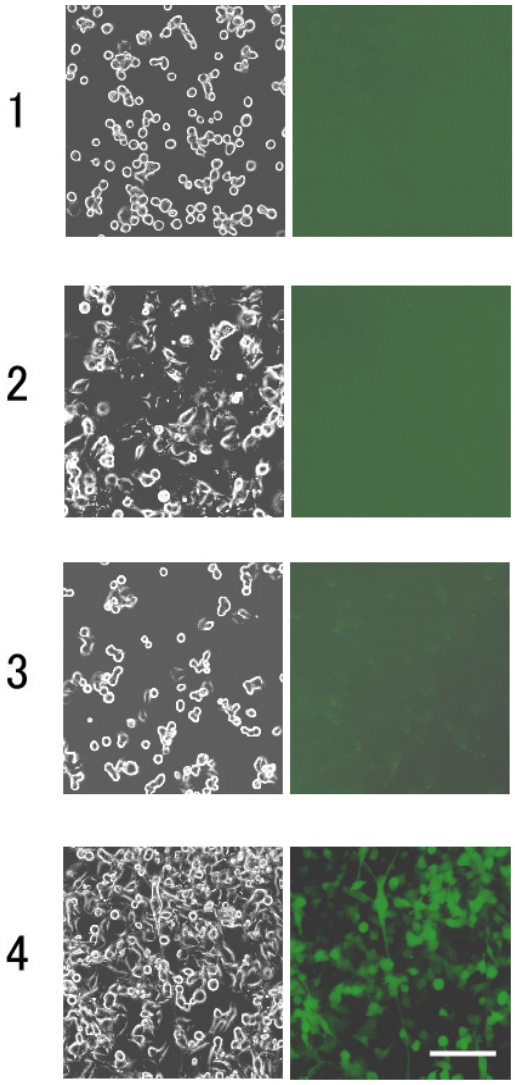

Fig. 5. Activation of HSP promoter in response to heat shock. EGFP expression in HDP-ND2 cells at 24 h after applying heat shock. Untransfected cells and HSP-ND2 cells without heat shock did not express EGFP (1-3). Heat shock applied HSP-ND2 cells expressed EGFP (4).

Induction of neural differentiation in response to electrical stimulation

To confirm that electrical stimulation can also induce neural differentiation mediated by recombinant NeuroD2 expression, HSP-ND2 cells were cultured on the ITO electrode surface. ITO electrode was treated with 1 N NaOH to make it hydrophilic in advance. There are no differences in cellular morphology and proliferation rate between cells on the ITO electrode and normal culture plate. After a day culture, cells were stimulated by a 100 Hz sine wave potential for which the amplitude was kept at +0.4 ~ -0.4 V versus the rest potential of electrode for 90 min.

At 24 h after the electrical stimulation, HSP-ND2 cells have started to exhibit EGFP fluorescence. On the other hand, cells without the electrical stimulation had not exhibited. These results prove that HSP promoter was activated in response to electrical stimulation. Moreover, at 72 h after the electrical stimulation, these EGFP fluorescent cells have elongated neurite similar at heat shock. On the contrary, control cells without electrical stimulation exhibited neither EGFP fluorescence nor neurite elongation (Fig.6). It confirmed that neural differentiation was caused in response to electrical stimulation as in the case of heat shock.

4. Discussion

Cellular gene expression modulate mechanisms are triggered by extracellular stimulations. And we can regulate conditional gene expression by utilize these cellular responsive mechanism. In this study we have tried to establish cells, which could differentiate into mature neural cells in response to conditional gene expression of recombinant NeuroD2 that activated by electrical stimulation.

It is known that tissue specific transcription factors are important for cellular differentiation. Some of them have ability to determine the cell fates. NeuroD2, neural specific bHLH transcription factor, is considered as an essential one for the neural differentiation. And it has been shown that transient expression of NeuroD2 has ability to differentiate into neurons from pluripotent mouse embryonal carcinoma cells [2]. We initially transfected pND2-IRES2-EGFP into mouse neuroblastoma cell line, N1E-115, to confirm the ability of NeuroD2 as a determinant factor of neural differentiation. As shown Fig.1 transiently transfected cells exhibited EGFP fluorescence and took neural morphology at the 5 days later. This fact suggests that conditional expression of NeuroD2 was able to induce neural differentiation in N1E-115.

We utilized HSP promoter to regulate conditional gene expression. The HSP promoter is activated not only heat shock, but also various physical stresses. Since electrical stimulation can be applied directly to cells and will be able to control easily compared with other physical stresses, we adapted electrical stimulation to activate the HSP promoter. However, the detailed mechanism of promoter response is unknown, Fig.4 and Fig.6 suggest that cells activate recombinant HSP promoter in response to electrical stimulation. Our previous study showed that the intracellular Ca2+ concentration increased and protein kinase C (PKC) was activated in the electrically stimulated PC12 cells [6].

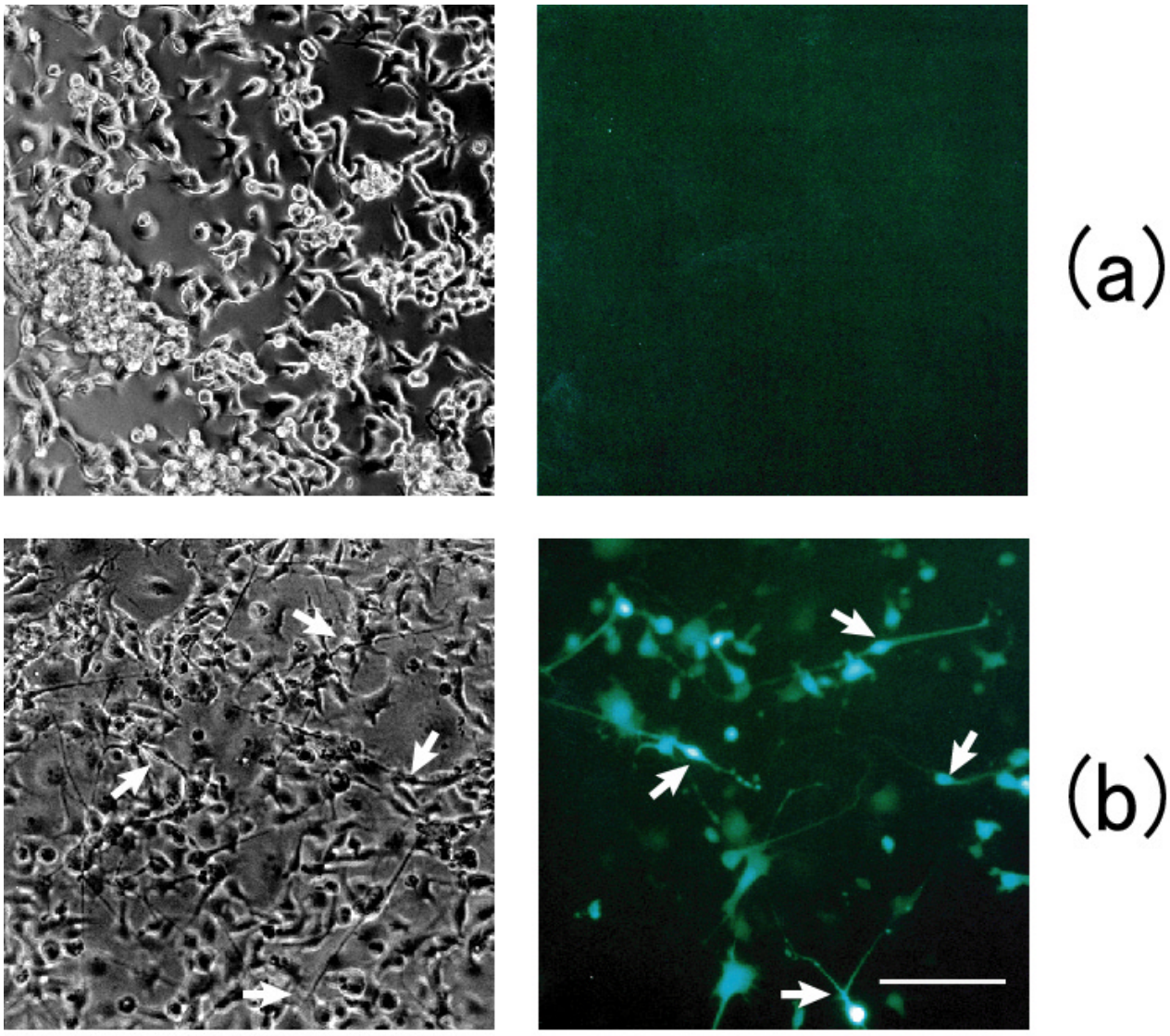

Fig. 6. Induction of neural differentiation by electrical stimulation. The photographs of cells 72 h after electrical stimulation. HSP-ND2 cells without stimulation did not express EGFP and maintained un differentiated state (a). Stimulated cells expressed EGFP and adopted neural morphology (b). The arrows indicate differentiated cells. Scale bar 200 μm.

And it is shown that PKC and Ca2+ enhanced heat shock factor 1 (HSF) thorough phosphorylation [7]. When the HSF is phosphorylated, it is translocated to the nuclei and enhanced HSP protein synthesis. Therefore, we think that electrically stimulated HSP promoter activity was induced by PKC or calcium dependent pathway via HSF.

As shown in Fig.6, electrically stimulated cells expressed EGFP fluorescence, which indicates expression of NeuroD2, and many of EGFP fluorescent cells exhibited neurite elongation. This result suggests that HSP-ND2 cells have ability to differentiate into neurons in response to electrical stimulation.

One of our aims is induction of neural differentiation in required places by using this system and cells. It would be a useful method for making neural networks as-ordered *in vitro*, which is helpful for understanding neural networks. In future, using extracellular recording techniques, it will be feasible to apply electrical stimulation to cells to make neural networks and to record neural communication on the microelectronic devices.

Reference

1. McCormick M.B. et al. (1996) NeuroD2 and enuroD3: distinct expression patterns and transcriptional activation potentials within the neuroD gene family. *Mol. Cell. Biol.* **16**, 5792-800
2. Farah M.H. et al. (2000) Generation of neurons by transient expression of neural bHLH proteins in mammalian cells. *Development* **127**, 693-702
3. Benjamin I.J. et al. (1990) Activation of the heat shock transcription factor by hypoxia in mammalian cells. *Proc. Natl. Acad. Sci. USA* **87**, 6263-7
4. Lin H. et al. (1999) A magnetic fieldresponsive domain in the human HSP70 promoter. *J. Cell. Biochem.* **75**, 170-6
5. Yanagida Y. et al. (2000) Electrically stimulated induction of hsp70 gene expression in mouse astroglia and fibroblast cells. *J. Biotechnol.* **79**, 53-61
6. Kimura K. et al. (1998) Differentiation and apoptosis of murine neuroblastoma cells N1E115. *Biochem. Biophys. Res. Commun.* **91**, 1309-18
7. Ding X. Z. et al. (1996) Increases in HSF1 translocation and synthesis in human epidermoid A-431 cells: role of protein kinase C and [Ca2+]i. *J. Inv. Med.* **44**, 144-53